EXPERIMENTAL CHOLERA PRODUCED BY CHOLERAGENIC FILTRATES: LOCALIZATION OF TOXINS BY FLUORESCENT ANTIBODY TECHNIQUES

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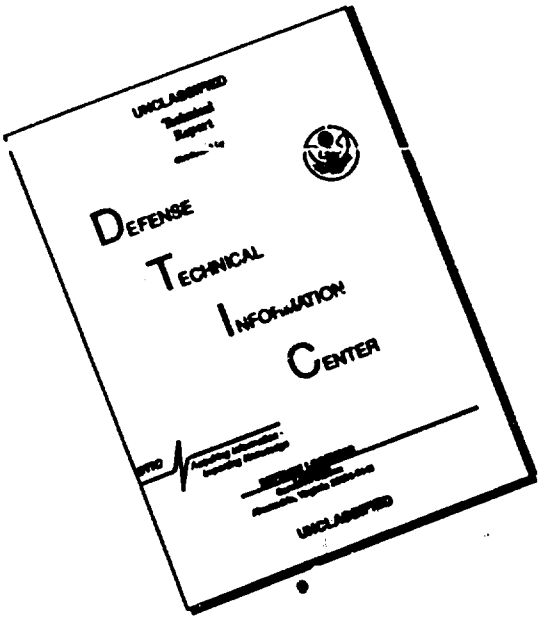

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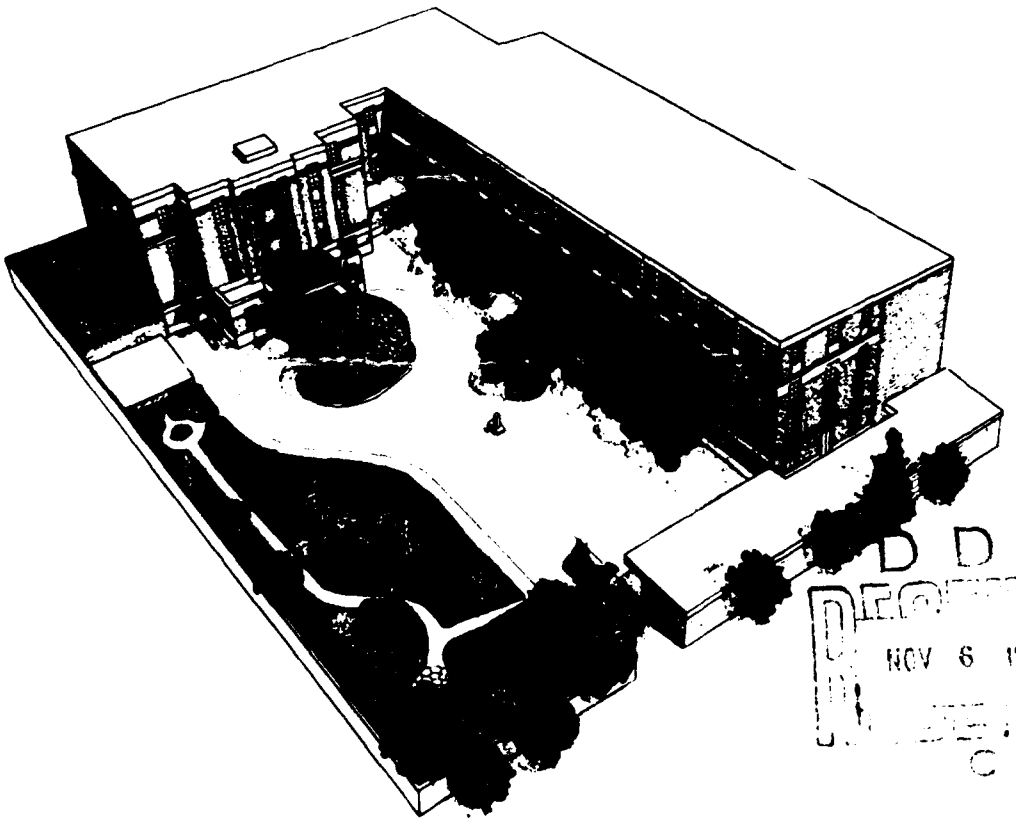

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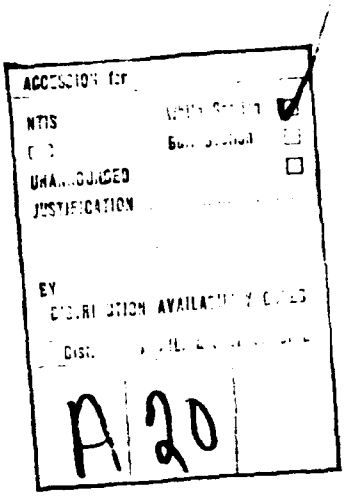

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13. ABSTRACT

EXPERIMENTAL CHOLERA WAS INDUCED BY FEEDING FILTRATES OF V. CHOLERAE BROTH CULTURE TO SUCKLING RABBITS THROUGH GASTRIC TUBES. THE DISSEMINATION OF CHOLERA METABOLITES CONTAINING BOTH EXOTOXIN AND ENDOTOXIN WAS THEN TRACED BY FLUORESCENT ANTIBODY TECHNIQUES AT DIFFERENT TIME INTERVALS AFTER ADMINISTRATION. A PATTERN OF DISTRIBUTION OF THE TOXINS INDICATING VARIOUS PHASES AND RATES OF ABSORPTION THROUGH INTESTINAL MUCOSA WAS DEMONSTRATED. THE BACTERIAL TOXINS PASSED THROUGH THE BRUSH BORDER OF INTESTINAL VILLI AND INTO THE CELLS OF THE LINING EPITHELIUM. LATER THESE SUBSTANCES WERE DETECTED IN DEEPER PORTIONS OF THE MUCOSA AND SUBMUCOSA AND IN THE WALLS OF SMALL BLOOD VESSELS. THEY PENETRATED THE VESSEL WALL BARRIERS ACHIEVING SYSTEMIC DISTRIBUTION. FLUORESCENCE APPEARED PREDOMINANTLY IN THE KIDNEY AND LUNG BUT LESSER AMOUNTS WERE FOUND IN THE HEART, LIVER, AND SPLEEN. FLUORESCENCE IN THE KIDNEY WAS FIRST DEMONSTRATED IN THE TUBULAR LUMENS AND WAS LATER SEEN ABSORBED ONTO THE TUBULAR EPITHELIAL CELLS. THE PRESENCE OF FLUORESCENT COMPLEXES IN HEART, LIVER AND LUNG TISSUE CORRELATES WITH HISTOLOGIC FINDINGS OF FOCAL MYOCARDITIS, HEPATIC PARENCHYMAL DEGENERATION, AND PNEUMONITIS.

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Chinese Journal of Microbiology 5: 27-35 (1972)

Experimental Cholera Produced by Choleragenic Filtrates: Localization of Toxins by Fluorescent Antibody Techniques

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Experimental cholera was induced by feeding filtrates of *V. Cholerae* broth culture to suckling rabbits through gastric tubes. The dissemination of cholera metabolites containing both exotoxin and endotoxin was then traced by fluorescent antibody techniques at different time intervals after administration. A pattern of distribution of the toxins indicating various phases and rates of absorption through intestinal mucosa was demonstrated. The bacterial toxins passed through the brush border of intestinal villi and into the cells of the lining epithelium. Later these substances were detected in deeper portions of the mucosa and submucosa and in the walls of small blood vessels. They penetrated the vessel wall barriers achieving systemic distribution. Fluorescence appeared predominantly in the kidney and lung but lesser amounts were found in the heart, liver, and spleen. Fluorescence in the kidney was first demonstrated in the tubular lumens and was later seen absorbed onto the tubular epithelial cells. The presence of fluorescent complexes in heart, liver and lung tissue correlates with histologic findings of focal myocarditis, hepatic parenchymal degeneration, and pneumonitis.

The development of profound diarrhea in cholera infection is believed due to the effect of bacterial toxin on the intestinal mucosa[10,8,2]. An additional factor may also be important in the infant rabbit model. Although the cholera vibrio does not invade host tissues, recent evidence suggests dissemination of some substances in the blood[5,11]. It is now possible, with fluorescent antibody techniques, to detect sites to which potentially toxic bacterial metabolites may be distributed. The purpose of this experiment is to describe appearance of potentially toxic materials in viscera of infant rabbits inoculated intraluminally with crude cholera broth filtrate and to evaluate possible effects by correlation with histologic alterations in this model.

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The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large.

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Material and Methods

Bacterial cultures

Vibrio cholerae strain 569B (Inaba) maintained on BHI slants was incubated overnight in peptone-NaCl broth adjusted to pH 8 with KOH[9].

Toxin preparation

Broth supernate from the bacterial preparations was filtered through a 0.22 μ millipore filter and concentrated with Biogel P-2 (BioRad Labs). The toxin was refrigerated at 4°C until used. Preparations of this type would be expected to contain the bacterial metabolites including both endotoxin and exotoxin[2].

Experimental animals

Twenty New Zealand white rabbits, 11 days old, weighing approximately 100–150 g, were fasted for 24 hours and their stomachs were lavaged via a No. 8 pediatric tube with warm, sterile bicarbonate saline solution (pH 8). Three ml of concentrated cholera broth supernate were given through the tube, then followed immediately with 2 ml sterile normal saline. To study sequentially the appearance of toxins in the viscera, 3 experimental animals were sacrificed and autopsied at 3, 6, 9, 12 and 15 hours after administration of the culture filtrate. Five animals served as controls.

Toxin localization

Anticholera toxin sera were obtained from 6 guinea pigs immunized by two subcutaneous injections of 1.0 ml filtered cholera culture medium with equal amounts of incomplete Freund's adjuvant at 2-week intervals. Control antiserum was prepared in the same manner by use of the peptone-NaCl broth alone. Both direct and indirect fluorescent antibody techniques were employed. Globulin fractions were separated by treatment of the whole sera with ammonium sulfate and labeled with fluorescein isothiocyanate by means of standard procedures[6].

Fluorescein-labeled rabbit anti-guinea pig gamma globulin serum was applied by indirect fluorescent techniques with use of hyperimmunized guinea pig antiseara according to a modified method of Cherry et al.[3] Controls for the specificity of the reaction included negative staining of the tissues from normal and control animals and negative staining of tissues following treatment with nonspecific guinea pig antiserum.

Tissue sections of ileum, kidney, heart, lung, liver, and spleen were sectioned on a cryostat microtome and were kept in slide boxes at –60°C until stained. An American Optical Fluorochrome ultraviolet microscope was used for examination and photomicrography.

Histologic examination

Portions of the viscera were fixed in 10% buffered formalin and processed by conventional histologic techniques with hematoxylin and eosin stains.

Results

General observations

The animals following challenge with cholera culture filtrate appeared severely ill and survived only a maximum of 15 hours. Complete autopsies of these animals at different time intervals revealed that the intestinal tracts contained turbid fluid and that the other viscera were pale.

Fluorescent microscopy

Small intestine. When sections of ileum from animals sacrificed at different time intervals were examined by fluorescent antibody methods a definite pattern of distribution of the cholera bacterial culture filtrate containing both exo- and endotoxins,

indicating various phases of the toxin absorption through the intestinal mucosa, was noted. At 3 and 6 hours after challenge strong fluorescence was observed along the surfaces of villi and crypt glands (Fig. 1). The filtrate appeared to be absorbed mostly onto the brush border. Absorption of the toxins into epithelial cells of crypt glands was noted; whereas, the cytoplasm of globular-shaped cells showed bright fluorescence (Fig. 1). Distribution of the toxins was limited to the upper layer of the mucosa. By 9 hours post-challenge the specific antigenic material was observed penetrating to the deep mucosa and submucosa (Fig. 2). There was spotty distribution of bright fluorescence in the cells of crypt glands, and clumps of bright fluorescence disposed in circular patterns indicated perivascular permeation in the layer of submucosa. Faint fluorescence still remained on the surface of the glands. Residual patchy fluorescence in the crypt glands was still visualized at 15 hours (Fig. 3).

Kidney. Early appearance of the cholera metabolic filtrates was noted in the lumens of collecting tubules by 3 and 6 hours after challenge (Fig. 4). At 6 hours the toxin was detected in the tubular cells of the lower segments and it was later found in the cells of the proximal and distal tubules at 9 and 15 hours (Fig. 5). The fluorescent complexes were sparsely scattered in these areas.

Lung. Sparsely disseminated, cytoplasmic, and patchy fluorescence was first observed in the alveolar septa at 6 hours. There was a consistently increased deposition of fluorescent bound toxins in the alveolar walls with discernible thickening by an increase of cellular elements in the later periods of the experiment (Fig. 6).

Heart. The appearance of spotty fluorescence in the cytoplasm of muscle fibers was demonstrated late, at 12 and 15 hours (Fig. 7).

Liver and spleen. Patchy fluorescence was occasionally seen in the hepatic and Kupffer cells 6 hours after challenge and persisted throughout the period of the experiment (Fig. 8). There were variable numbers of fluorescent positive cells observed in the red pulps of spleens 9 hours after challenge.

Light microscopy

Small intestine. Mild histologic alterations of intestinal mucosa were observed at different periods of time after challenge. At 3 and 6 hours regressive changes of epithelial lining cells were noted, manifested by cytoplasmic vacuolization and nuclear dispolarity. Mild edema and slight vascular congestion were present in the lamina propria and submucosa. By 9, 12, and 15 hours, fraying of the epithelial lining cells and widening of the lamina propria and submucosal space with fluid and cellular exudates were observed, while the vacuoles in the lining cells disappeared.

Kidney. The histologic changes involved principally the limbs of Henle loops and collecting tubules at 6 hours postchallenge and later at 9, 12, and 15 hours extended to proximal and distal tubules. Early there was hydropic swelling consisting of cytoplasmic vacuolization with intact nuclei (Fig. 9). All the vessels were distended with erythrocytes, some of which had extravasated. Cellular infiltration was not seen. Further progressive degenerative changes of the tubular cells were observed in the proximal and distal tubules, characterized by cytoplasmic hyalinization and swelling with obliteration of the tubular lumens, while hyaline casts were found in some of the dilated tubules. No appreciable histologic changes occurred in glomeruli.

Lung. Progressive patchy pneumonitis observed in the later periods of the experiment was characterized by septal widening with swelling of capillary endothelial cells associated with cellular infiltrates. Capillary congestion was remarkable, while some of red blood cells had extravasated into the alveolar spaces.

Heart. Histologic alteration consisting of cytoplasmic vacuolization and loss of normal striation occurred early, by 6 hours. Later, muscle fiber dissolution occurred with cellular infiltration in focal distribution (Fig. 10). Foci of myocardial hemorrhage were also present. No histologic changes were seen in either endocardium or epicardium.

Liver and spleen. Focal parenchymal hepatic cell degeneration featuring increased cytoplasmic eosinophilia and nuclear pyknosis was present at 15 hours. No significant histological changes in the parenchyma of the spleen were observed.

The findings of both fluorescent and light microscopic observations are summarized in Table 1 and 2. The results in visualization of the bacterial metabolites by fluorescent antibody techniques were consistent in both direct and indirect methods. The loss of these substances in aqueous media during the procedures appeared minimal after proper prefixation of the tissue sections.

Discussion

The results of the present investigation are consistent with previous studies of experimental cholera[4,5]. Absorption and systemic circulation of bacterial products occurred following inoculation of the small intestine of the infant rabbit with sterile broth filtrates of *V. cholerae* cultures. The absorbed products may include cholera toxin(s) and further work is required to trace the fate of purified toxin preparations in this model.

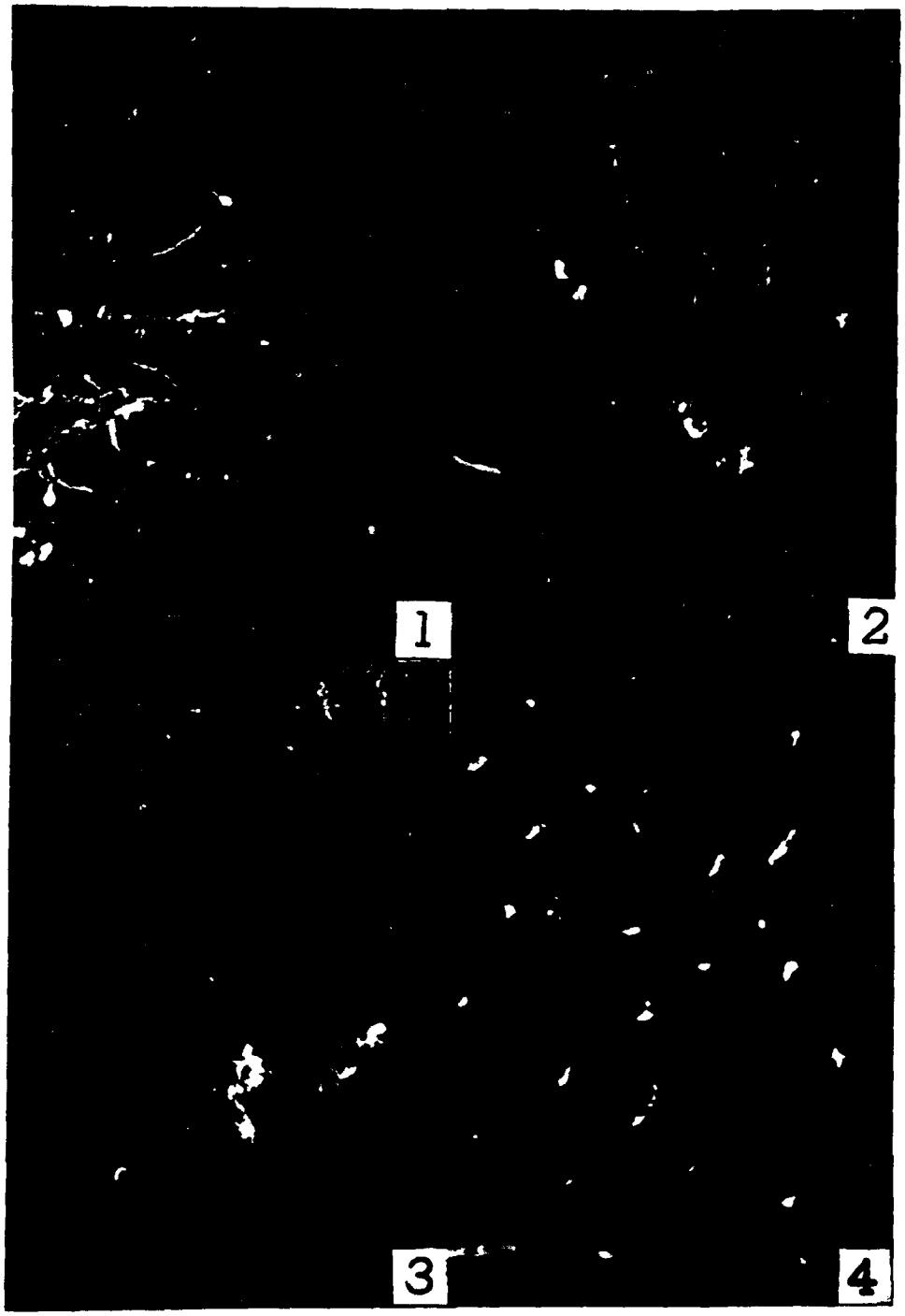
Also demonstrated was a pathway of absorption commencing at the brush border of intestinal lining epithelium and proceeding into the depths of the mucosa and submucosa. The bacterial metabolites then accumulated around small blood vessels and penetrated the walls to enter the systemic circulation. The disseminated products were found in different tissues at different rates and in a sequential manner.

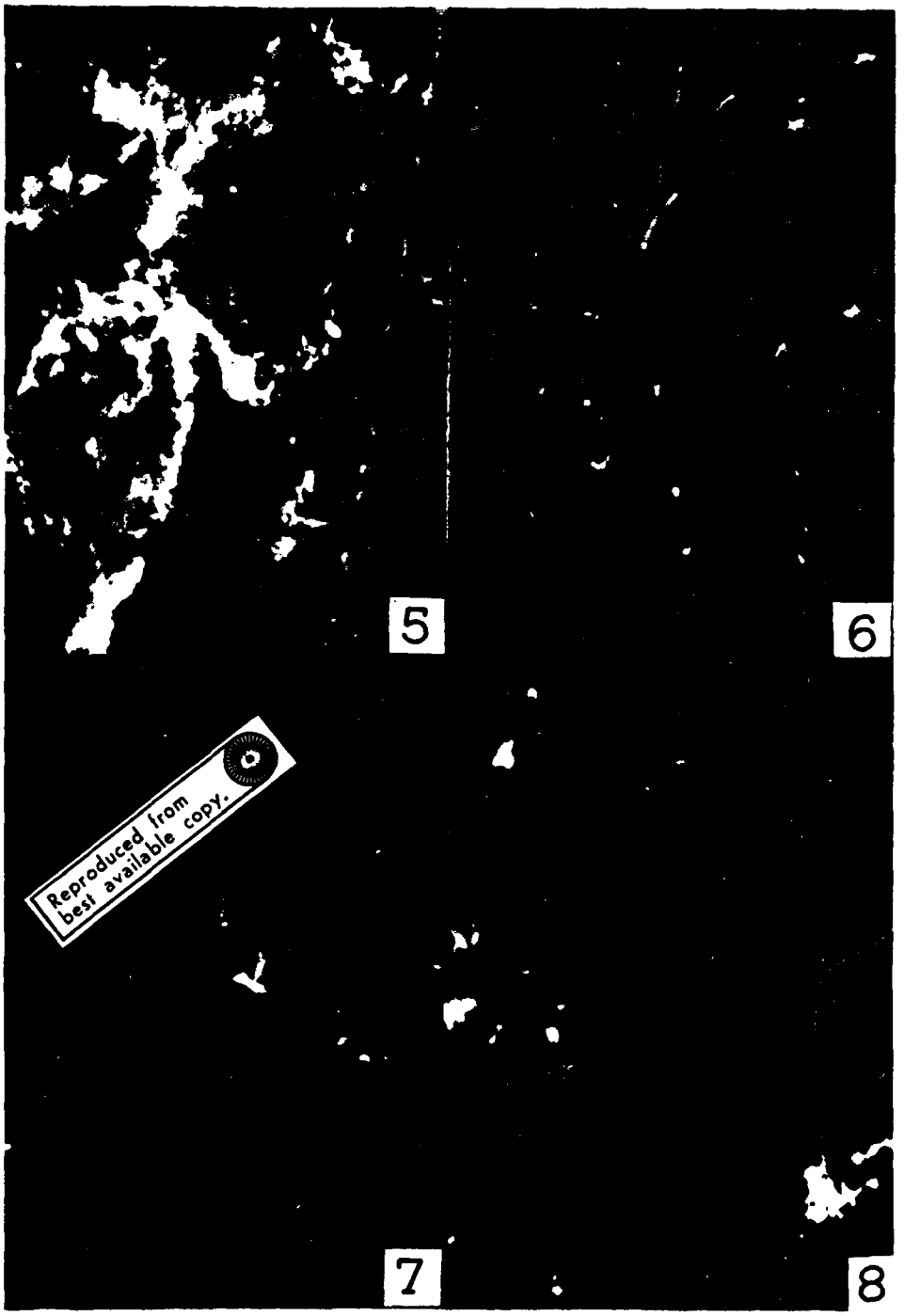
If cholera toxin was among the absorbed metabolites it may have affected the endothelial cells of venules and capillaries within the lamina propria and submucosa[4]. The localization of fluorescence in the walls of vessels in the submucosal layer suggested a possible direct action of toxin on blood vessels and provided evidence of toxin absorption through the blood vessel walls.

Observed clinical cases of cholera have indicated the importance of the kidney in the host response in relation to the severity of the disease[1]. The status of renal failure as a result of acute tubular nephrosis may be affected by toxic metabolites as well as by electolyte imbalance and fluid loss. The kidney may play a protective role in the early course of toxemia since the fluorescing substances are excreted by the kidney as observed in the lumens of lower segmental tubules 3 hours after challenge. Similar features in experimental staphylococcal entero-toxemia are reported by Norman et al. indicating that the kidney is the predominant site of early toxin localization[7]. Whether cholera toxins have an important effect on renal function in the infant rabbit model remains to be determined.

The liver is known to prevent toxins from gaining access to other distant organs. Cholera metabolites were present in the liver parenchyma and could be visualized by fluorescent antibody techniques and, additionally, parenchymal liver cell degeneration was prominent in animals after challenge with the broth filtrates.

Cytoplasmic vacuolization of myocardial fibers appeared early at 3 and 6 hours following filtrate administration and fluorescence was visualized in spotty patterns in the cytoplasm of myocytes, whereas focal myolytic lesions with inflammatory cell infiltration occurred at 12 and 15 hours. This may indicate a direct effect of





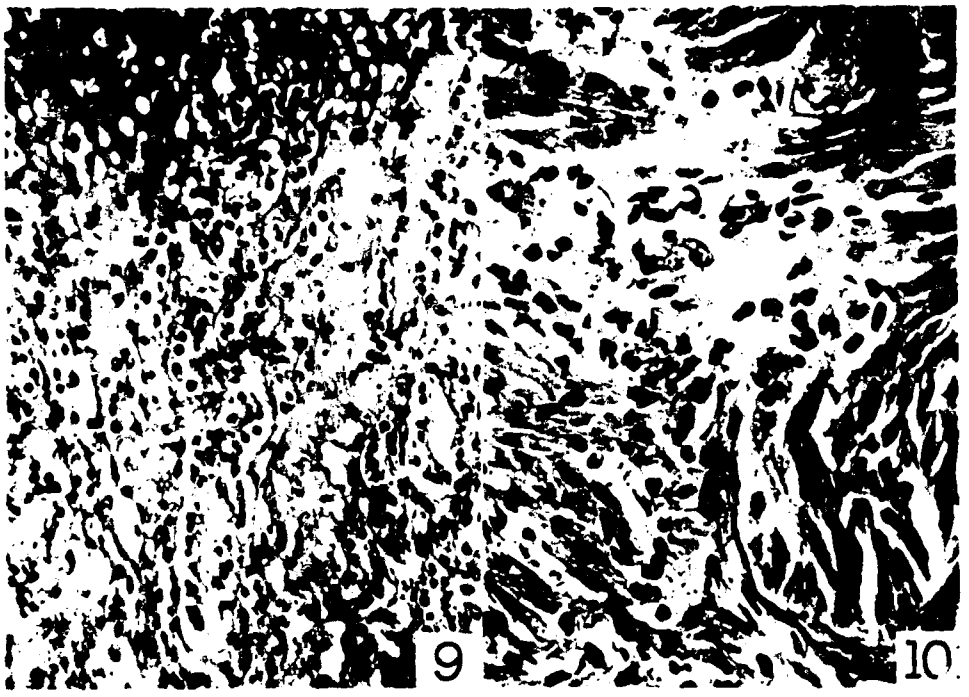


Figure 1. Ileum of an infant rabbit 3 hours after challenge. Fluorescing cholera toxins are seen along the surfaces of villi and inside the cells of crypt glands. Indirect fluorescent antibody stain (IFAS), ×129.

Figure 2. Ileum of an infant rabbit 9 hours postchallenge. The cholera toxins as shown by bright fluorescence are penetrating to the deep mucosa and to the perivascular areas in the submucosa. IFAS, ×129.

Figure 3. Ileum of an infant rabbit 15 hours postchallenge. Residual fluorescence in the crypt glands is still visualized. IFAS, ×240.

Figure 4. Kidney of an infant rabbit 3 hours postchallenge. The brightly fluorescent toxins are shown in the lumens of collecting tubules. IFAS, ×240.

Figure 5. Kidney of an infant rabbit 6 hours after challenge. The toxins appear in the cells of tubular epithelium of lower segments. IFAS, ×240.

Figure 6. Lung of an infant rabbit 9 hours postchallenge. The bright fluorescence is demonstrated in the cells of alveolar walls. IFAS, ×240.

Figure 7. Myocardium of an infant rabbit 12 hours after challenge. Fluorescein bound toxins are observed in the cytoplasm of myocytes. IFAS, ×480.

Figure 8. Liver of an infant rabbit 6 hours postchallenge. Spotty fluorescence is demonstrated in the parenchymal cells. IFAS, ×480.

Figure 9. Kidney of an infant rabbit 6 hours postchallenge. The tubular epithelium of lower nephrons shows hydropic swelling and cytoplasmic vacuolization. H & E, ×240.

Figure 10. Myocardium of an infant rabbit 12 hours postchallenge. A focus of muscle fiber dissolution is present with cellular infiltration. H & E, ×480.

Table 1. Fluorescent and light microscopic findings of small intestine and kidney affected by cholera toxins in infant rabbits

Hours after challenge	Small Intestine				Kidney			
	Fluorescent microscopy			General histologic findings	Fluorescent microscopy			General histologic findings
	Brush border	Crypt glands	Lamina propria & submucosa		Tubular lumen	Tubular cells of lower segments	Tubular cells of proximal & distal	
3	2+	1+		Cytoplasmic vacuolization of epithelial lining cells	2+			No significant finding
6	2+	2+		Edema & vascular congestion in lamina propria	1+	1+		Vacuolization & hyalinization of tubular epithelium in lower nephron
9	1+	1+	2+	Fraying of surface of epithelial cells and cell infiltration in lamina propria		1+	2	Epithelium cell swelling with obliteration of tubular lumens
12		1+	1+	Marked edema and congestion in lamina propria and submucosa		1	1+	Same as above
15		1+		Same as above		1+	1+	Same as above

Table 2. Fluorescent and light microscopic findings of heart, lung, liver, and spleen affected by cholera toxins in infant rabbits

Hours after challenge	Heart	Myocardium	Lung	Alveolar septa	Liver	Hepatic cells	Spleen	Red pulps
	Fluorescent microscopy	General histology	Fluorescent microscopy	General histology	Fluorescent microscopy	General histology	Fluorescent microscopy	General histology
3	-	Vacuolization & hyalinization of muscle fibers		No significant finding		No significant finding		No significant finding
6		Same as above	1+	Capillary congestion	1+			
9	-	Same as above	2+	Thickening by cell infiltration	1+	-	1	-
12	1	Muscle fiber dissolution with cell infiltration	2+	Same as above	1+		1+	-
15	1+	Same as above	2+	Same as above	1+	Eosinophilic degeneration	1+	

cholera metabolites on heart muscle encountered in the course of infection.

The findings of cellular and patchy fluorescence in the alveolar septa could indicate a precipitating cause for the pneumonitis observed. Aspiration during filtrate administration could also account for the presence of fluorescence.

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幼兔霍亂病實驗研究—霍亂菌毒之腸內吸收過程與內臟中分佈部位觀察

美國海軍第二醫學研究所病理科微生物科及榮民總醫院研究部

B. T. Schaeffer, 孫詩潛 R. I. Walker

（1971年5月25日受理）

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